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Preparation and Use of Transplantable Cell Line of Newborn Rabbits for Reproduction of Viruses.

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ABSTRACT

Despite many preventive antiviral preparations, the epizootic situation by viral diseases of animals still remains intense. Therefore, need of improvement of biotechnological process of receiving a cellular substratum and raw virus is actual for the development of highly effective vaccines. The purpose of the real researches was development of a way of receiving culture of cells from bodies of newborn rabbits for a reproduction of production strains of viruses. Researches are executed on 60 newborn rabbits of breed "Chinchilla" weighing 10-50 g of 1-30-day age. At the first stage of work conducted researches on receiving cultures of cells of kidneys of rabbits 1-2-day, 3-7-, 8-10-, 11-20- and 21-30-day age. The research was carried out on 60 newborn rabbits breed "Chinchilla" weighing 10-50 g 1-30 days of age. At the 1st stage of the work was carried out research on the preparation of kidney cell cultures rabbits 1-2 days, 3-7-, 8-10-,11-20 and 21-30 days of age. From anesthetized rabbits were removed kidneys, crushed with scissors to 1-3 mm and after washing them with saline Hanks, incubated at 37 °C 30-40 minutes followed by treatment in 0.25 % solution of trypsin, and by centrifugation of the cells at 1000-1500 rpm for 10 min. After removal of the supernatant centrifugate resuspendable in growth medium eagle (MEM), after we determined the number of cells in the camera Goryaeva at the light microscope and the cells were seeded on a specified culture medium. To get the most pure population of cells used the adhesive method by pouring the mixture homogenized and trypsinization cell growth medium and culturing them for 24 h with later transfer is not implanted cells to new culture medium for further selection. As a result of this two-fold purification was obtained by primary culture of kidney cells of newborn rabbits (KPNC). According to the method described in the process were obtained 10 different primary cell cultures (PC) of kidney from rabbits of different ages: PK-1 (donor age -1 day), PC-2 (2 days), PC-3 (5days), PC-4 (7days), PC-5 (10 days), PC-6 (15 days) PK-7 (20 days) PK-8 (25 days), PC-9 (30 days), PC-10 (35 days). It is found that complete mono layer formation of cells from donors 1-2 -day-old stepped on 3-4 days of cultivation, while from donors of other ages (7-,10-,25-, 30-, 35 - days of age) results of the primary cultures were unsatisfactory. In the second phase of work carried out studies to find the optimal limits of cultivation of a cell line KPNC. It is established that the optimal parameters of cultivation of this cell line are: sowing concentration of 3-4.105 cells/cm3, the filling volume of the vessel 25%, pH 7,2-7,4; cultivation temperature of 37±0,50 C; duration of cultivation 48-72 h. the Results of cytogenetic studies derived cell line KPNC showed their stability in the karyotype: modal class are 44 chromosomes, its value is 50%, the variation of the number of chromosomes in the cells from 39 to 48. Results virological studies showed that the obtained cell line KPNC sensitive to parvovirus in cattle, the herpes virus type 1 virus PG-3, the virus VD-BS and Euske disease virus and the possibility of reproduction of these viruses to receive on the basis of their antiviral preparation.

Keywords: immortalized cell lines; requirements for cellular substrates; virus reproduction; viral vaccines; primary culture cells; kidney newborn rabbits; virus.

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INTRODUCTION

Modern biotechnology is entering a new qualitative level. In industrial scale production mastered dozens of different genetically engineered biotherapeutic drugs [4]. Traditional technology of production of viral vaccines on the basis of primary and diploid cell cultures and can no longer fully meet the growing requirements of practical medicine and veterinary medicine in an efficient, safe and economical medicines. In an alternative cell substrate are considered transplantable cell line (PLC), which are the most promising for the production of Biopharmaceuticals [17].

The development of cellular technology have allowed widely use of cells of different animal species in virus studies [23].

The active use of primary kidney cell cultures of embryos and cows (PEC), bovine testicle (BT) and light or subcultures, as well as transplantable cell lines (MDC) in Virology and emerging new aspects of their application broadens the spectrum of cell lines with different properties [9,14]. Primary cell cultures obtained from both whole embryos and individual organs and tissues of newborn and adult animals. The results of the comparative studies showed that of all examined organs and tissues are most preferred for the cultivation of the viruses were cell cultures from the renal tissue [20, 22]. It is established that cells of embryonic origin, and newborn animals usually remain viable longer than those obtained from the tissues of adult animals [1].

Currently, the best biotechnology for production of viral mass is a primary cell culture of rabbit 25-35 days of age [13]. Although this method is considered the best for producing viral biomass, but it has several disadvantages: 1) with increasing age of rabbits 35 days, the degree of coverage within 14 days of monolayer reduced by 25-75%; 2) increased passage of submultiframe there was a decrease of sensitivity to viruses, and 11 - passage culture becomes insensitive to them; 3) culture cells up to passage 6 are more sensitive to the virus myxomatosis of rabbits than cultures of more than 6 passages and even fixed line RK-13; 4) growing rabbits up to 28-35 days of age implies additional economic costs for the care and maintenance of animals, which leads to increase in the cost of production. Based on these data, our studies were aimed at improving the technology for primary trypsinization culture of kidney cells of newborn rabbits.

MATERIALS AND METHODS

The research was carried out on 60 newborn rabbits breed "Chinchilla" 1-30 days of age live weight of 10-50 g. At the appropriate time after birth, the rabbits were euthanized ether anesthesia, kidneys were removed, removed the capsule, and crushed with scissors into pieces of 1-3 mm in size. The pieces of tissue were carefully cleaned from the remnants of blood with saline Hanks and subjected to disaggregation of 0.25% solution of trypsin. Trypsinization was simultaneous with pre-incubation of slices with 370C and exposure 30-40 min. After the specified exposure, the cells were centrifuged at 1000-1500 rpm. After centrifugation the supernatant was decanted and centrifuged resuspendable in the nutrient medium. As nutrient media used eagle (MEM), 199, 0,5% solution of hydrolyzed lactalbumin (GLA) and fetal serum. The number of cells in primary trypsinization the suspensions were determined by standard technique using Goryaev's chamber in a light microscope. Sowing dose primary trypsinization of kidney cells of newborn rabbits was $3\text{-}4.10^5$ cells/cm3. To obtain the most pure population of cells used a technique based on the different adhesive abilities of cells [21]. This mixture of cells obtained by mechanical and enzymatic treatment of renal tissue, were seeded in the growth medium and cultivated 24 h, then carefully implanted cells were transferred to a new culture medium for further selection. Primary trypsinization cells were grown in the hospital environment in glass vials with a capacity of 250 ml, bottles Carrel (diameter 55 mm), 24 - 6 - hole plastic plates for cell culture, the vial of penicillin. Cells were separated from the glass mixture (1:1) of 0.25 % solution of trypsin and 0.02 % solution of Versene at room temperature. The proliferative activity of the cultures was assessed using the index of proliferation [6]. The viability of cells in suspension was determined using a 0.5 % aqueous solution of trypan blue.

Cryopreservation as primary trypsinization and transplantable cells was performed according to the standard scheme [5]. Preparations for routine cytogenetic studies of primary cell lines were prepared according to the standard technique [26].

Painting of chromosome preparations was performed using ready-to-dye azure-eosin by Romanovsky. Control cell cultures for sterility was carried out by plating on bacteriological medium: MPA, MPB, Saburo, kit-Tarozzi.

Virological methods we determined the sensitivity of cell cultures of the kidneys of newborn rabbits to the parvovirus cattle type I (IRT) (vaccine strain TK-A (VIEW) - 2" parainfluenza virus-3 (strain SF-4"), virus diarrhea - diseases of the mucous membranes (strain "VK-1"), the virus Aujeszky's disease (strain "Arsky"). Titers of virus were calculated by the method of [27] and expressed in log CFU/ml, log LD 50/ml and log TCD 50/ml, respectively.

Statistical processing of results was performed on a personal computer using the Statistica 8 program. The accuracy of the value changes of the parameters were determined by non-parametric criterion of Wilcoxon.

RESULTS OF RESEARCHES

Using the above techniques by successive passages over 75 passages we have obtained 10 primary cells of the kidneys of rabbits of different ages that were conventionally marked PC-1 (donor age 1 day), PC-2 (2 d), PC-3 (3сут), PC-4 (7сут), PC-5 (10-day), PC-6 (day 15), PK-7 (20 days) PK-8(day 25), PK-9 (30 days), PC-10 (35 days). The results of testing the obtained variants of the cell cultures according to the degree of monolayer formation are presented in table 1.

Table 1: The degree of monolayer formation of primary cells of rabbits of different age

Primary cell culture	Donor age (days)	The resulting primary culture	Monolayer	
			the degree of formation (%)	time (days)
PC-1	1	+	100	3
PC-2	2	+	100	4
PC-3	5	+	80	4
PC-4	7	-	0	14
PC-5	10	-	0	8
PC-6	15	+	65	14
PC-7	20	-	0	14
PC-8	25	-	0	14
PC-9	30	+	25	14
PC-10	35	-	0	14

It is established that with increasing age of donor cells increased the time of incubation and decreased the degree of monolayer formation. Thus, in the investigation it was determined the optimal donor age for obtaining primary trypsinization KNC, which was 1-2 days after the birth of the young rabbits. Further conducted the experiments to obtain the most pure population of cells by cleaning their method, based on the different adhesive abilities of cells. Obtained by mechanical and enzymatic treatment of renal tissue, a cell culture was filled with growth medium and cultured 24 h. Then the attached cells are gently transferred to new culture medium for further selection. The result is two-fold purification was obtained by primary culture of kidney cells of newborn rabbits.

In the next phase of work carried out research for obtaining a transplantable cell line kidney of rabbits. For this primary trypsinization culture cells obtained by the above procedure, successively passaged in the optimum for this cell line the growth medium - eagle (MEM) for 75 passages. At the level of 60-65 passages marked stabilization of growth and cytomorphological characteristics of the cells. Bacteriological monitoring showed that this cell line does not contain bacterial and fungal contaminants. Thus, in result of the conducted researches we obtained transplantable cell line kidney of newborn rabbits-KNK. Further conducted research on the adaptation of continuous cell line cell line KPNC when growing monoclonal method. Obtained a line of cells within 10 passages adapted to monoclona method of cultivation Py in mattresses capacity of 1.5 L. Since the adaptation of continuous cell line cell line when grown monoclonal method requires optimal conditions of cultivation, we were tested by varying the parameters: the content of serum of cattle (cattle) in the medium Needle MEME from 5 to 15%, the crop concentration in the range of 2-5.105 cells/cm3, pH of from 7.2 to 7.4,

temperature of 36 to 39 0C. Criteria of optimisation of the cultivation parameters were the maximum cell concentration in the medium and the index of proliferation (IP). The results of these studies are presented in table 2.

Table 2: Optimum process parameters monolingo cultivation of cell lines KPNC

Figure	Parameter values
Sowing concentration (cells/ml)	$3\text{-}4x10^5$
The filling volume of the vessel (%)	25
Cultivation temperature (0C)	37±0,5
the pH of the medium	7,2-7,4
The total duration of cultivation (hour)	48-72

The study of proliferative stability obtained in transplantable cell line CPNC under these optimized culture conditions was carried out for 10 consecutive passages. The results of the study are presented in table 3.

Table 3: Proliferative characteristics of cells KPNC in the process of continuous cultivation monolingo

Passages cells	Sowing concentration (cells/ml)	The density of the cell monolayer after 72 hours (cells/ml)	The index of proliferation	Viability (%)
1-5	$3,2x10^5$	$6,2x10^5$	2,1	90
6-10	$4,4x10^5$	$5,8x10^5$	1,8	87

In the next step work carried out the karyological study of the stability of the cells KPNC at stationary cultivation. This takes into account that one of the main criteria of stability of the biological properties of cell populations is the persistence of the karyotype of the cells during continuous cultivation. Karyological analysis was subjected to primary trypsinization culture of kidney cells of newborn rabbits, and obtained transplantable cell line KPNC after the stabilization of its biotechnological characteristics at 75 passage monolingo continuous cultivation in the optimized process conditions. Carried out according to standard methods karyological analysis of the primary trypsinization cell cultures KPNC showed that the number of chromosomes in the cells ranged from 39 to 48, and the modal class is represented by cells with 44 chromosomes. At 75 passage KPNC karyological analysis showed a slight decrease in the value of modal class (50%) while maintaining its number (44 chromosomes) and interval of variation of chromosome number in cells (от39 to 48).

In General, it should be noted the high level of karyological stability of primary trypsinization culture of kidney cells of newborn rabbits and the resulting cell lines KPNC during long-term cultivation. On the basis of the results of cell culture KPNK can be attributed to the group of cell lines stable karyotype.

At the final stage of the work was carried out studies on the sensitivity of transplantable cell line KNC to different viruses. Virusprogram the ability of transplantable cell line kidney of newborn rabbits KPNC at 75 passage for respiratory and intestinal viruses of cattle was determined by passage of herpesvirus type 1, parainfluenza virus-3, viral diarrhea virus, and the virus of Aujeszky's disease at the cellular monolayer. In the experiments used a virus PG-3 (strain SF-4") with hemagglutination titer of 1:32, the herpes virus type-1 (strain TK-A (VIEW) B2 cattle with infectious titer of 7.0 lg TCD50/ml, the virus Aujeszky's disease (strain "Arsky"), viral diarrhea virus-diseases of the mucous membranes (strain "VK-1").

The herpes virus, type 1 was cultivated in monolayer culture KPNC. With each subsequent passage in monolayer cell titer of the virus increased, and at the third passage were consistent with lg TCD 50 ml=7,5±0,1. The titer of the virus PG-3 on the third passage corresponded to a 1:32 titer of virus diarrhea-disease of mucous membranes (VD-BS) match lg TCID50/ML= 6,25±0,1. Against the virus, Aujeszky's disease characteristic cytopathic effect was observed after 72 h after inoculation of the culture of this strain, which allows to make a conclusion that the vaccine strain "Arsky" virus Aujeszky's disease is able to proliferate in cell culture KPNC. The obtained data allow to conclude that culture KPNK can be used for reproduction of viruses herpesvirus type 1 virus (parainfluenza-3) PG-3, virus VD-BS (viral diarrhea) virus Aujeszky's disease to the accumulation of viral mass.

Conducted in the present study in experiments on cell cultures obtained from rabbits of different ages, comparisons on the proliferative, cytomorphological and karyological characteristics, showed significant differences of the studied parameters. The choice of renal tissue was caused by easy disaggregation of the tissue with detergents, the relative simplicity of cells to culture conditions, a wide spectrum of sensitivity to viruses of animals and humans [3]. In this case, it is preferable to obtain biological material from newborn animals, because it contains more progenital cells, and also has a lower immunogenicity than the cells and tissues of adults [21].

Given the above, we have obtained 10 options KPNC 1-30 days of age. The results of comparative experiments for assessing the degree of monolayer formation by kidney cells of donors of different ages showed that with increasing age of donors increased and incubation time decreased the degree of monolayer formation. It is established that the optimal donor age for obtaining primary trypsinization culture of kidney cells is 1-2 days.

Improving the effectiveness of the method of producing CPNC in our research was addressed through a significant reduction in the age of the donors (up to 48 hours of life), which leads to a significant reduction of production costs with simultaneous increase in the sensitivity of cell cultures to viruses and yield of virus mass (Patent RU №2520868, 2013).

In the process of developing large-scale technologies of cultivation of cells and viruses is necessary to consider a number of factors influencing the achievement of the ultimate goal. Thus, the primary task is creation of favorable conditions for the cells as close as possible in vivo. This is achieved by optimizing the main parameters: sowing cell concentration, multiplicity of infection cells, filling volume of the bioreactor, maintaining a level of pH and temperature during cultivation [4,11].

Given the above, the next step of the research was carried out adaptation of transplantable cell line CPNC to the stationary conditions of cultivation. The results of screening studies with different variation of content in growth medium of one of the most important components of culture media - serum of cattle, showed that it had the highest trophic efficiency at 10% rated the content in the cell culture medium KNK that is associated with the intensification of metabolic processes and the optimum content of biologically active substances in the environment [10].

Given that the verification of the effective concentrations of sowing is a critical link in optimizing the system for cultivating cells [12], conducted research to determine the optimal seeding concentration of the cells KPNC in the stationary cultivation. It is established that the maximal accumulation of cells is achieved by using 3.4.105 seeding concentration (cells/ml3, in which the maximum increase in the density of cells happens 2 times after 48 h, but after 72 h there was a decrease in their concentration. This is due to the fact that in the process of cell growth is the utilization of amino acids in the nutrient medium, leading to the inhibition of the rate and intensity of growth [2,6].

Cell line, cell cultures are not homogeneous by cytogenetic characteristics. The reason for the heterogeneity of cultures can be a source of cells obtained from body tissue of the host, also the mutation processes occurring in cells during the reproduction of culture in an unbalanced composition of the nutrient medium, Often the technical result of human error, possible cross-contamination with cells of different lines that happens quite often [7].

During prolonged cultivation of cells can accumulate chromosomal changes, changing the morphology of the cells and their metabolic characteristics. Karyological instability can lead to the loss of specific features of a given culture, changes in morphological and growth properties of the cells [8,15].

We obtained the data you should make a conclusion on high level of karyological stability of primary trypsinization culture of kidney cells of newborn rabbits and the resulting cell lines KPNC during long-term cultivation, despite the change of culture media, serum, as well as the changing methods of cultivation. According to the karyological analysis, the cells remain stable - the number of chromosomes in the cells ranges from 39 to 48, and the modal class is represented by cells with 44 chromosomes (the value of the modal class is 50%).

Based on the karyological analysis of the cell culture KPNK can be attributed to the group of cell lines stable karyotype [16].

At the final stage of the work carried out studies to determine the sensitivity of the cell line KPNC to viruses: in relation to the IRT viruses, PG-3, VD-BS and to the virus Aujeszky's disease.

When carrying out virologic studies, we assumed that viral infections are the leading in the world among other infectious pathologies in humans and animals. Reproduction of viruses of man, animals and birds in sensitive cell lines is the basis of obtaining antigens for vaccine production. Virus culture also helps to solve a number of theoretical problems associated with the study of the interactions of the "virus-cell" [18, 29]. In addition, a number of applied problems related to the diagnostics and production of drugs for the prevention of viral infection is impossible without the accumulation virusdatabase materials [28]. For cultivation of viruses, you can use fabric of any kind.

The above has led researchers to the use of primary cell cultures as the most similar to the conditions in vivo model system to study the various properties of various viruses [15].

The virological results of the study showed that the herpes virus type-I were cultured in a monolayer, its cytopathic effect was characterized by a specific JRS. With each subsequent passage in monolayer cell titer of the virus increased and on the third passage was lg TCID50/ml =7,5±0,1, which allows to make a conclusion that the cell culture has a pronounced KNK virusbarrieruser activity and has the possibility of using for reproduction herpesvirus type I. cell Culture KPNC virusbarrieruser has a more pronounced activity against the virus PG-3 than cell culture MDBK and has a strong possibility to be used for the accumulation of viral mass. The results of studies to determine the sensitivity of cell cultures to the diarrhea virus VD-BS showed that the first and second passages, the titer of virus in the culture MDBK was higher and was 4.5 and 5.5 respectively, but the third passage virus titer was increased to lg TCID50/ml = 6,25±0,1, which allows to make a conclusion that the cell culture KNK can also be used for reproduction of the virus VD-BS. Vaccine strain "Arsky" virus Aujeszky's disease is able to proliferate in cell culture KPNC. Characteristic of tSPD was observed 72 hours after infection culture this strain.

Thus, the obtained cell line KPNC is active against the virus PG-3, Aujeszky's disease virus VD-BS, the herpes virus type I.

CONCLUSION

The method of selection obtained a new monoclona transplantable cell line kidney of newborn rabbits (KPNC) with a proliferation index of 2.1±0,09 in the medium Needle MEM with 10 % content of blood serum of cattle or 5% blood serum of fetuses of cows; stable karyotype: modal class are 44 chromosomes, and its value is 50%, the variation of the number of chromosomes in the cells from 39 to 48. Optimal culturing conditions of primary trypsinization culture of kidney cells of newborn rabbits (with medium Needle MEM with 10% serum of adult cattle, 5% serum fruits cows). Growth, cytomorphological properties and karyological characteristics of the resulting line KNK for at least 10 continuous cycles of cultivation at the stationary exhaust technological modes are stable. Determined the sensitivity of culture of kidney cells of newborn rabbits to the parvovirus in cattle, the herpes virus type 1 virus PG-3 VD-BS and the virus of Aujeszky's disease and the possibility of their reproduction.

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